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Dapdiamides, Tripeptide Antibiotics Formed by Unconventional Amide Ligases[†]

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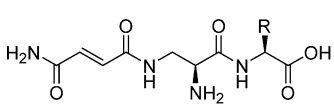
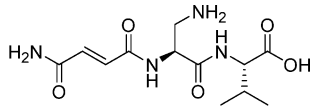
Construction of a genomic DNA library from *Pantoea agglomerans* strain CU0119 and screening against the plant pathogen *Erwinia amylovora* yielded a new family of antibiotics, dapdiamides A−E (**1**−**5**). The structures were established through 2D-NMR experiments and mass spectrometry, as well as the synthesis of dapdiamide A (**1**). Transposon mutagenesis of the active cosmid allowed identification of the biosynthetic gene cluster. The dapdiamide family's promiscuous biosynthetic pathway contains two unconventional amide ligases that are predicted to couple its constituent monomers.

Condensation reactions, the joining of two molecules with the formal loss of water, dominate the biosynthesis of large and small biological molecules. In nonribosomal peptide synthetase (NRPS) pathways, condensation domains that are evolutionarily related to the coenzyme A-dependent acyltransferases used in primary metabolism carry out repetitive condensations between amino, hydroxy, and aryl acid monomers.[1] A recent study on the andrimid biosynthetic gene cluster from *Pantoea agglomerans* and other bacteria led to the realization that transglutaminase-like enzymes also carry out biosynthetic condensations.[2,3] In this report, we describe another antibiotic family from *P. agglomerans* (**1**−**5**) in which an L-2,3-diaminopropionic acid fragment (DAP) is linked to two variable units through two amide bonds. The family's promiscuous biosynthetic pathway contains no NRPS condensation domains.



Dapdiamide A (1), R = i-Pr
Dapdiamide B (2), R = s-Bu
Dapdiamide C (3), R = i-Bu



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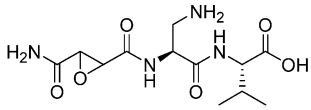

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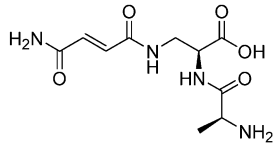

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Results and Discussion

Both the dapdiamide family members and their biosynthetic gene cluster were captured by constructing a genomic library of *P. agglomerans* strain CU0119 and screening for cosmid clones active against *Erwinia amylovora*. Strains of *P. agglomerans* (formerly *E. herbicola*) have previously been shown to produce numerous antibiotics active against *E. amylovora*, the causative agent of the devastating plant disease fire blight.[4,5] A subclone of a dapdiamide-producing cosmid was transposon mutagenized to identify the biosynthetic genes. In this way, a cluster of nine ORFs from the cosmid insert was identified, and it was further subcloned into pUC19 and transformed into *E. coli*. From this plasmid subclone (pUC19 A10A) members of the dapdiamide family were isolated using bioassay-guided fractionation and linked to their biosynthetic gene cluster.

From the ^{1}H NMR spectra of the active fractions it was apparent that several structurally related compounds were responsible for the antibiotic activity against *E. amylovora*. The high-resolution MS spectrum of the most abundant compound (**1**) exhibited an $[M + H]^+$ ion at *m/z* 301.1517, indicating the molecular formula $C_{12}H_{20}N_4O_5 + H$. In a DQCOSY spectrum of **1**, three proton−proton spin systems corresponding to fumaramic acid, L-2,3-diaminopropionate (DAP), and L-valine units were evident (Figure 1). HMQC and HMBC 2D NMR experiments (Table 1) demonstrated that fumaramic acid is joined by an amide bond to the β-amino group of DAP, while Val is linked through an amide bond to the DAP carboxyl. Several less abundant structural variants of dapdiamide A were also isolated: dapdiamides B (**2**) and C (**3**) contain isoleucine and leucine instead of valine (Table 1); dapdiamide D (**4**) is identical to **1** except the linkage between fumaramic acid and DAP is through the α-amino group of DAP; and dapdiamide E (**5**) is identical to **4** but has an epoxide in place of the fumaramic acid double bond (Table 2).

In order to determine the absolute configuration of the natural product, dapdiamide A (**1**) was synthesized with the L configuration at both DAP and Val alpha carbons (Figure 2). The ^{1}H and 2D NMR data of synthetic dapdiamide A were identical to dapdiamide A isolated from A10A. The specific rotation $[\alpha]^{23}_D$ for isolated dapdiamide A was +5.9 and that for synthetic dapdiamide A was +9.5 in water. Circular dichroism spectra of synthetic and isolated dapdiamide A indicate that the configuration of the two compounds is the same (Figure 3).

The most likely annotation of the gene cluster for dapdiamide biosynthesis (Figure 4) identifies two genes responsible for producing DAP (blue), four genes involved in the biosynthesis of fumaramic acid (red), two genes responsible for amide bond formation (green), and a gene for self-resistance (yellow). DdaAB are homologous to enzymes previously implicated in DAP biosynthesis[6] and will not be discussed further here.

Biosynthesis of Fumaramic Acid (DdaCDEH). The pathway for fumaramic acid biosynthesis likely begins with succinate. Like other NRPS pathways that generate oxidatively modified building blocks,[7] the dapdiamide gene cluster encodes an adenylation−thiolation (A-T) enzyme, DdaD, that sequesters the monomer to be oxidized. The A domain of DdaD is predicted to activate succinate by adenylation and then tether it to the adjacent DdaD thiolation domain as a thioester. This sequestered monomer would be oxidized by DdaC, a putative Fe(II)/α-ketoglutarate-dependent dioxygenase.

[†] Dedicated to the late Dr. John W. Daly of NIDDK, NIH, Bethesda, Maryland, and to the late Dr. Richard E. Moore of the University of Hawaii at Manoa for their pioneering work on bioactive natural products.

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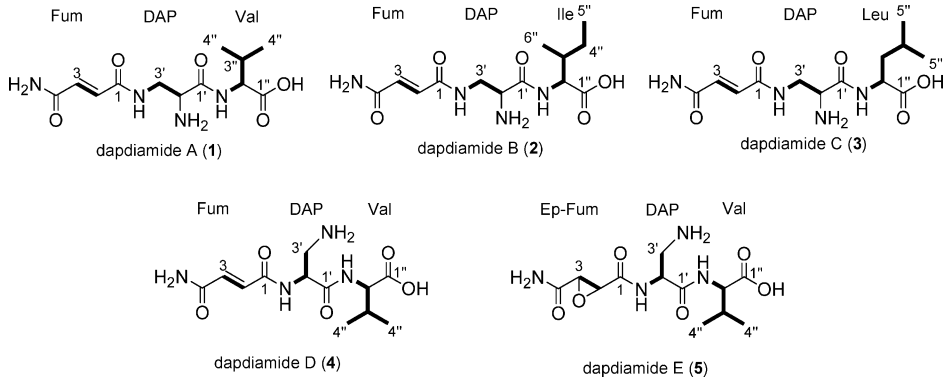

Figure 1. DQCOSY correlations for the dapdiamides are indicated with bold lines.

Table 1. NMR Spectroscopic Data (600 MHz, DMSO-d_6) for Dapdiamides A−C (**1**−**3**)

	dapdiamide A (**1**)			dapdiamide B (**2**)			dapdiamide C (**3**)		
position	δ_C[a], mult.	δ_H (*J* in Hz)	HMBC[b]	δ_C[a], mult.	δ_H (*J* in Hz)	HMBC[b]	δ_C[a], mult.	δ_H (*J* in Hz)	HMBC[b]
Fum									
C1	164.3, qC			164.6, qC			164.3, qC		
C2	132.3, CH	6.78, d (15.2)	1, 3, 4	132.5, CH	6.78, d (15.2)	1,3,4	132.2, CH	6.78, d (15.2)	1, 3, 4
C3	133.1, CH	6.85, d (15.2)	1, 2, 4	133.3, CH	6.84, d (15.3)	1,2,4	133.1, CH	6.84, d (15.2	1, 2, 4
C4	164.9, qC			165.2, qC			164.9, qC		
4a-NH		7.34, s	3, 4		7.34, s	3,4		7.34, br s	
4b-NH		7.80, s	4		7.80, s	4		7.81, br s	
DAP									
C1′	166.7, qC			166.8, qC					
C2′	51.5, CH	4.06, t (5.9)	1′, 3′	51.8, CH	4.03, t (5.9)	1′, 3′	51.6, CH	3.96, m	
2′-NH_2		8.22, br s			8.13, br s				
C3a′	39.5, CH_2	3.52, m	1, 1′, 2′	39.8, CH_2	3.53 (m)	1, 1′, 2′	39.7, CH_2	3.55, t	
C3b′		3.56, m	1, 1′, 2′						
3′-NH		8.44, t (5.9)	1, 3′		8.44, t (5.8)	1, 3′			
Val/Ile/Leu									
C1″	172.2, qC			172.5, qC					
1″-OH		12.95, br s							
C2″	57.2, CH	4.24, dd (5.2, 8.3)	1′, 1″, 3″, 4a″, 4b″	56.6, CH	4.28, dd (5.2, 8.1)	1′, 1″, 3″, 4″, 6″	50.3, CH	4.28, dd (7.8, 14.7)	
2″-NH		8.68, d (8.4)	1″, 2″		8.68, d (7.9)	1′, 2″		8.79, br d (7.6)	
C3″	29.7, CH	2.12, m	1″, 2″, 4a″, 4b″	36.6, CH	1.82, m	2″, 4″, 5″, 6″	39.5, CH_2	1.56, m	5a″
C4a″	17.5, CH_3	0.91, d (7.2)	2″, 3″, 4b″	24.5, CH	1.21, m	2″, 3″, 5″, 6″	23.9, CH	1.67, m	
C4b″	18.8, CH_3	0.92, d (7.2)	2″, 3″, 4a″	24.5, CH	1.43, m	2″, 3″, 5″, 6″			
C5a″				11.2, CH_3	0.87, t	3″, 4″,	20.9, CH_3	0.87, d (6.5)	3″, 4a″, 5b″
C5b″ or C6″				15.4, CH_3	0.89, d (7.0)	2″, 3″, 4″	22.6, CH_3	0.91, d (6.6)	3″, 4a″, 5a″

[a] ^{13}C NMR chemical shifts are assigned from $^1H-^{13}C$ HMQC and HMBC correlations. [b] HMBC correlations are from proton(s) stated to the indicated carbon.

Enzymes from this family catalyze oxidative modifications of unactivated carbon centers, including hydroxylation, desaturation, and epoxide formation.[8] In the case of the dapdiamides, DdaC typically catalyzes the formation of a double bond, but may occasionally catalyze an additional two-electron oxidation to form an epoxide instead. There is precedent for the formation of an epoxide from a hydroxy group by another enzyme from this class, HppE.[9] Finally, the resulting oxidized succinate derivative is likely released from DdaD by DdaE, a homologue of the type II thioesterase domains that cleave acyl groups from thiolation domains.[10] DdaH, a homologue of asparagine synthetase, should transaminate one of the carboxylic acids of the succinate-based monomer, but the timing of its action cannot be predicted bioinformatically.[11,12]

Amide Bond Formation (DdaFG). The dapdiamide gene cluster does not encode any enzymes known to form amide bonds in secondary metabolic pathways. However, it encodes two proteins with sequence homology to enzymes that activate carboxylate monomers in primary metabolic pathways: DdaF, a member of the ATP-grasp superfamily, and DdaG, a homologue of phenylacetate-CoA ligase. DdaF is homologous to biotin carboxylase and phosphoribosylglycinamide synthetase, both of which catalyze the formation of amide bonds via an acyl phosphate intermediate. Therefore, DdaF likely catalyzes one of the two condensation reactions needed to join the dapdiamide monomers. Related ATP-grasp enzymes have been shown to form intramolecular ester and amide cross-links in microviridin and related cyanobacterial natural products, hinting at a wider role for this enzyme superfamily in natural product biosynthesis.[13,14]

The other amide bond is likely formed by DdaG, a distant homologue of phenylacetate-CoA ligase, which activates the carboxylic acid of phenylacetate by adenylation for the subsequent attack of the terminal thiol of coenzyme A. DdaG may similarly activate a carboxylic acid by adenylation, perhaps for direct amide bond formation without the intermediacy of a CoA-linked thioester species. While acyl-CoA ligase enzymes are not known to catalyze subsequent condensation reactions, DdaG has an unassigned ~100 amino acid domain at its C-terminus that may be involved in positioning an amine nucleophile for attack.

Self-Resistance (DdaI). Antibiotic biosynthetic pathways typically include a mechanism for resistance to the antibiotic being produced. Plasmid-borne DdaI, a predicted transmembrane efflux pump, conferred resistance to **1** in an otherwise sensitive *E. coli* strain, confirming its role as a dapdiamide resistance gene.

The target of β-linked *N*-fumaramoyl-DAP has previously been described as glucosamine-6-phosphate synthase.[15] This enzyme

Table 2. NMR Spectroscopic Data for Dapdiamides D (**4**) (600 MHz, DMSO-d_6) and Dapdiamide E (**5**) 600 MHz, D_2O)

	dapdiamide D (**4**)			dapdiamide E (**5**)		
position	δ_C[a], mult.	δ_H (J in Hz)	HMBC[b]	δ_C[a], mult.	δ_H (J in Hz)	HMBC[b]
Fum						
C1	164.5, qC			169.1, qC		
C2	132.4, CH	6.85, d (15.4)	1, 3, 4	53.7, CH	3.82, d (2.0)	1
C3	133.3, CH	6.86, d (15.3)	1, 2, 4	53.6, CH	3.75, d (2.0)	4
C4	165.1, qC			170.9, qC		
4a-NH		7.36, br s	3			
4b-NH		7.83, br s				
DAP						
C1′	168.5, qC			169.4, qC		
C2′	50.4, CH	4.72, d (4.7, 8.5, 8.5)	1, 1′, 3′	50.8, CH	4.89, dd (4.9, 8.5)	1
2′-NH		8.83, d (8.2)	1, 2′			
C3a′	39.9, CH_2	2.97, dd (9.7, 12.4)		40.0, CH_2	3.37, dd (4.5, 9.0)	1′, 2′
C3b′		3.23, dd (8.6, 13.1			3.55, dd (4.9, 13.5)	1′, 2′
3′-NH_2						
Val						
C1″	172.5, qC			175.9, qC		
C2″	57.3, CH	4.16, dd (5.6, 8.5)	1′, 1″, 3″, 4a″, 4b″	59.4, CH	4.35, d (5.7)	1′, 1″, 3″, 4a″
2″-NH		8.14, d (8.5)	1′			
C3″	29.7, CH	2.08, m	1′, 2″, 4a″, 4b″	30.0, CH	2.27, m	4a″, 4b″
C4a″	17.8, CH_3	0.86, d (6.8)	2″, 3″, 4b″	17.2, CH_3	0.98, d (7.0)	2″, 3″, 4b″
C4b″	18.9, CH_3	0.87, d (6.8)	2″, 3″, 4a″	18.6, CH_3	0.99, d (7.0)	2″, 3″, 4a″

[a] ^{13}C NMR chemical shifts are assigned from $^1H-^{13}C$ HMQC and HMBC correlations. [b] HMBC correlations are from proton(s) stated to the indicated carbon.

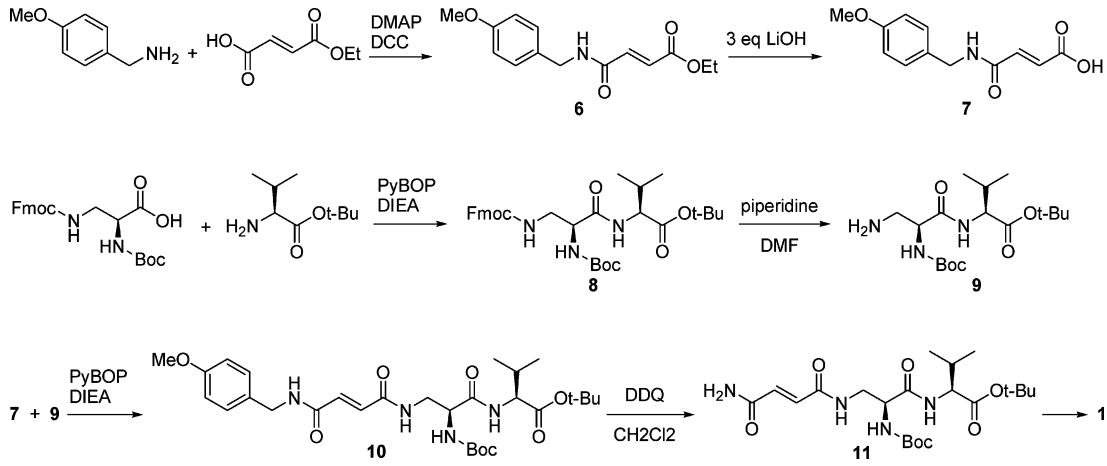

Figure 2. Synthesis of dapdiamide A (**1**). Details of yields and procedures are given in the Experimental Section.

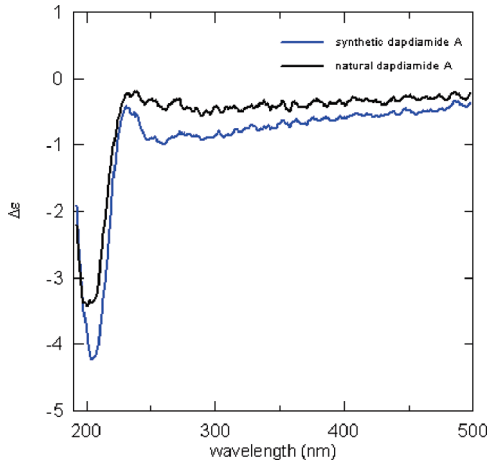

Figure 3. Circular dichroism of isolated (natural) and synthetic dapdiamide A (**1**).

converts D-fructose-6-phosphate to D-glucosamine-6-phosphate, a precursor to UDP-*N*-acetyl-D-glucosamine, an essential building block for bacterial cell wall biosynthesis.[16] The catalytic cysteine of the glutamine binding domain of glucosamine-6-phosphate synthase becomes inactivated when it attacks the double bond of the fumaramoyl moiety and becomes irreversibly attached.[17]

There are a few natural products structurally similar to the dapdiamides. CB-25-I, isolated from *Serratia plymuthica*, has a structure nearly identical to **1** but has an epoxide in place of the olefin.[18] While the biosynthetic gene cluster for CB-25-I has never been identified, a pathway nearly identical to the biosynthetic pathway for the dapdiamide compounds in GenBank from the *Serratia proteamaculans* 568 sequencing project (locus tags Spro_0339−Spro_0347) suggests that this pathway has been shared between these enterobacteria. Two actinomycete-derived compounds, A19009 and Sch37137 (the epoxide analogue of A19009), contain the *N*-fumaramoyl-DAP core but have L-alanine linked to DAP via the α-amino group.[19,20] These compounds could be made by the same pathway that uses an enzyme with different chemo- and regioselectivity for the formation of the second amide bond, but no sequence data are available. The isolation of such similar molecules from unrelated Gram-negative and Gram-positive bacteria suggests that the dapdiamide gene cluster is highly mobile, and the divergent structures of the dapdiamides A19009 and Sch37137

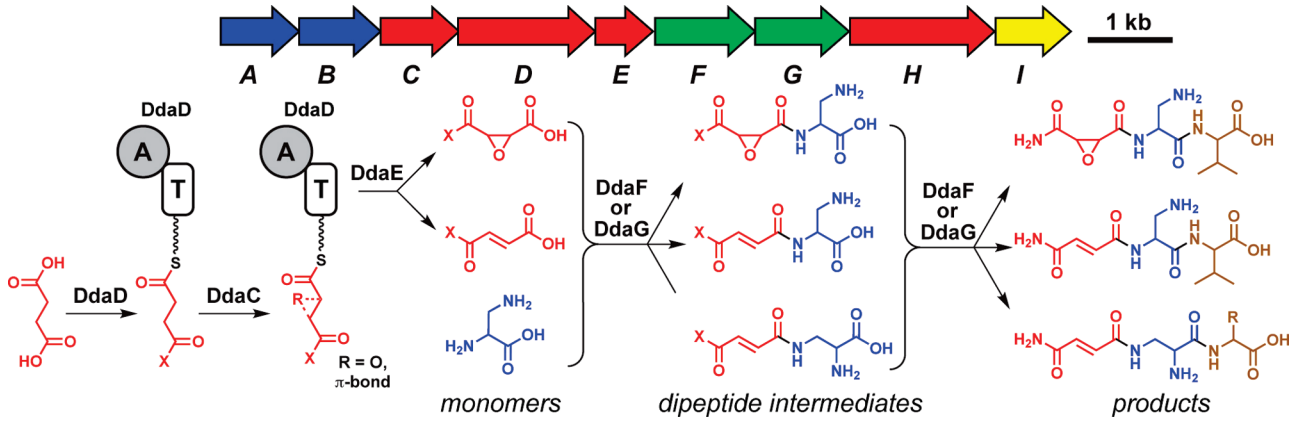

Figure 4. Gene cluster and proposed scheme for dapdiamide biosynthesis. The order of steps shown here is one of several possibilities.

are a testament to the plasticity of a pathway based on atypical condensation catalysts.

Experimental Section

General Experimental Procedures. CD spectra were obtained using an Aviv circular dichroism spectrometer model 202. NMR spectra were obtained using a Varian Inova 600 MHz NMR spectrometer. LC-MS spectra were obtained with a Micromass LCZ quadrupole mass spectrometer. High-resolution mass spectra were obtained with a Micromass Q-Tof2 mass spectrometer using reserpine as a lock mass. Samples were injected through the HPLC system, and the LC flow was combined with the flow of a reserpine solution from a syringe pump. Restriction enzymes were purchased from New England Biolabs. Plasmids were isolated using Qiaprep Spin Miniprep kits. All other reagents were purchased from Sigma-Aldrich unless otherwise indicated.

Culture Media and Strains. *Pantoea agglomerans* strain CU0119 and *Erwinia. amylovora* strain 273 were obtained from Professor Steven Beer at Cornell University. Cultures of *E. coli* were grown in Luria−Bertani (LB) media or on LB-agar plates. Antibiotics were used at the following concentrations: ampicillin (amp) 100 μg/mL; chloramphenicol (cm) 25 μg/mL; kanamycin (kan) 30 μg/mL. For blue/white screening 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was added to a final concentration of 80 μg/mL, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a concentration of 20 mM. Bioassays with *E. amylovora* were conducted using glucose-asparagine (GA) medium containing 20 g glucose, 0.3 g L-asparagine, 0.05 g nicotinic acid, 11.5 g K_2HPO_4, 4.5 g KH_2PO_4, and 0.12 g $MgSO_4$ per liter.[21] Cultures of *E. coli* with cosmids or plasmids containing the dapdiamide biosynthetic pathway were grown in *E. coli* minimal medium (EcMM) containing per liter 0.25 g yeast extract, 20 mL glycerol, 4.0 g K_2HPO_4, 1.72 g KH_2PO_4, 0.5 g NaCl, 2.0 g $(NH_4)_2SO_4$, 0.2 g sodium citrate, and 0.02 g $MgSO_4 \cdot 7H_2O$. Both GA and EcMM were sterilized by filtration through a 0.2 μm filter.

***Erwinia amylovora* Bioassays.** Bioassay plates were made by diluting an overnight culture of *E. amylovora* grown in LB into warm, sterile top agar (GA with 0.75% agar). To test *E. coli* transformants for activity, a small portion of a colony was patched onto the surface of the agar using a wooden stick. To test liquid samples for activity, 3 μL of each sample was pipetted directly onto the surface of the agar. Assay plates were incubated overnight at room temperature and then examined for zones of inhibition.

Cosmid Library Construction and Screening. *P. agglomerans* strain CU0119 exhibited a large zone of inhibition in *E. amylovora* bioassays, so it was selected for further study. A cosmid library of *P. agglomerans* strain CU0119 genomic DNA was constructed using the pWEB cosmid cloning kit from Epicenter according to the manufacturer's instructions. The cosmid clones were screened against *E. amylovora*, and one active cosmid clone, 119C1, was identified. The cosmid was isolated and retransformed into *E. coli*, and the antibiotic activity was confirmed by rescreening against *E. amylovora* on a GA agar plate.

Subcloning the Active Cosmid. The active cosmid and plasmid vector pBC were digested with NotI and ligated using the Fast-Link DNA ligation kit from Epicenter. The ligation mixture was transformed into electrocompetent DH10B *E. coli* cells (Invitrogen) and plated on LB plates containing cm, X-gal, and IPTG. White colonies were screened for antibiotic production in *E. amylovora* bioassays. An active subclone, p119C1.1, was identified, transformed into *E. coli* DH10B, and rescreened against *E. amylovora* to confirm the activity.

Transposon Mutagenesis of p119C1.1. To identify genes responsible for the biosynthesis of the antibiotics, the active plasmid (p119C1.1) was transposon mutagenized using the GPS-1 Genome Priming System from New England Biolabs according to the manufacturer's instructions. The mutagenized plasmids were transformed into DH10B cells, and transformants were screened against *E. amylovora* for loss of antibiotic production. Transposon insertion mutants that eliminated antibiotic production were mapped by sequencing out from the ends of the transposons using primers S and N, which are supplied with the GPS kit. DNA sequencing was performed at the Dana-Farber/Harvard Cancer Center DNA Resource Core in Boston, MA. The sequences obtained from the knockouts were assembled into a contiguous DNA sequence spanning roughly 14 kilobases. Using the ORF Finder on MacVector (Accelrys), a cluster of nine open reading frames was identified that had transposon insertions that knocked out antibiotic production.

Subcloning p119C1.1. To confirm that the cluster of nine open reading frames identified by transposon mutagenesis was indeed responsible for antibiotic production, this portion of p119C1.1 was subcloned. p119C1.1 was digested with DrdI, and the ~12 kb fragment containing the biosynthetic cluster was gel purified using a Qiaquick gel extraction kit (Qiagen). To form blunt ends, the End-It DNA end-repair kit (Epicenter) was used according to the manufacturer's instructions. The pUC19 vector was prepared by digestion with *Sma*I, dephosphorylation with calf intestinal alkaline phosphatase (CIP), and gel purification using the Qiaquick gel extraction kit. The DrdI fragment and *Sma*I-digested pUC19 were ligated using the Fast-Link DNA ligation kit according to the manufacturer's instructions, and the ligation reaction was transformed into electrocompetent EC100 *E. coli* cells (Epicenter). An active subclone, named A10A, was identified and verified by double restriction digest. A10A was checked for stability by growing cultures from several different colonies in EcMM and testing them for activity on GA plates seeded with *E. amylovora*. The construct consistently inhibited growth.

Isolation of Dapdiamides. A starter culture of *E. coli* subclone in pUC19 (A10A) was grown in 10 mL of LB amp overnight at 30 °C. Twenty 500 mL Erlenmeyer flasks containing 50 mL of EcMM media were inoculated with 150 μL of starter culture. The cultures were incubated at 30 °C for 6 h at 250 rpm and then incubated at room temperature for 16 h at 200 rpm. The cultures were combined and centrifuged for 25 min at 12000*g*. The culture supernatant was applied to a cation exchange column (AG 50W-X8 resin from Bio-Rad). Twenty 50 mL fractions were eluted with 500 mM ammonium hydroxide at a flow rate of 6 mL/min. The pH of each fraction was adjusted to 6.5−8.5 using acetic acid or ammonium hydroxide. Fractions were dried and assayed for activity against *E. amylovora*. The active fractions were combined, and this material (~1 g) was further purified by reversed-phase C-18 chromatography. Thirty 125 mL fractions were eluted with a MeOH/H_2O step gradient. The fractions active against *E. amylovora*

were combined except for the first active fraction, fraction 3, which was high in salt and was purified separately. Fraction 3 (~450 mg) was purified by HPLC using a reversed-phase C-30 semipreparative column with a flow rate of 2 mL/min and a H_2O/MeOH gradient (0−100% MeOH over 25 min). The remaining active fractions from the C-18 column, F4 to F16, were purified by HPLC on a semipreparative C-18 column with a flow rate of 4 mL/min using H_2O/MeOH acidified with 0.01% trifluoroacetic acid as the eluent (2.5−25% MeOH over 20 min). Approximately 3 mg of dapdiamide A, 1.4 mg of dapdiamide B, 1.6 mg of dapdiamide C, 1 mg of dapdiamide D, 1.5 mg of dapdiamide E, and 1.5 mg of *N*-fumaramoyl-DAP were isolated.

Synthesis of Dapdiamide A. (*E*)-Ethyl 4-(4-methoxybenzylamino)-4-oxobut-2-enoate (6). To form the fumaramic acid portion of dapdiamide A, we chose to make the *N*-(methoxybenzyl)fumaramic acid (**6**) derivative. Monoethyl fumaric acid (40 mmol) and 4-dimethylaminopyridine (DMAP) (44 mmol) were dissolved in CH_2Cl_2 (400 mL), and 4-methoxybenzylamine (4-MBA) (40 mmol) was added. *N*,*N*′-Dicyclohexylcarbodiimide (DCC) (44 mmol) dissolved in CH_2Cl_2 (100 mL) was added, and the mixture was stirred overnight. The precipitate from the reaction was removed by filtering and washed with CH_2Cl_2. The CH_2Cl_2 was extracted with sulfuric acid (0.5 M, 500 mL), then H_2O (500 mL), and then saturated aqueous $NaHCO_3$ (500 mL) and dried with sodium sulfate. The CH_2Cl_2 was evaporated and the product was further purified by silica flash chromatography eluting with EtOAc/hexanes (3:2). The fractions containing the product were combined and evaporated to afford **6** (7.83 g, 74%): TLC $R_f = 0.53$, EtOAc/hexanes (3:2); ^{1}H NMR (500 MHz, CD_3OD) δ 1.30 (3H, t, $J = 7.1$ Hz), 3.77 (3H, s), 4.24 (2H, q, $J = 7.1$ Hz), 4.39 (2H, s), 6.73 (1H, d, $J = 15.5$ Hz), 6.88 (2H, d, $J = 8.6$ Hz), 7.02 (1H, d, $J = 15.5$ Hz), 7.22 (2H, d, $J = 8.6$ Hz); LC(ESI)MS $m/z = 264$ $[M + H]^+$.

(*E*)-4-(4-Methoxybenzylamino)-4-oxobut-2-enoic acid (7). To form the acid of **6**, the ethyl group of **6** was hydrolyzed with lithium hydroxide by dissolving **6** (4 g, 15 mmol) in MeOH (50 mL) and adding 3 equiv of LiOH (46 mL of 1 M solution in water). After stirring at room temperature for 2 h the MeOH was evaporated using a rotary evaporator, 100 mL of water was added, the pH was adjusted to roughly 4 with citric acid (5% in water), and the water was extracted with EtOAc (450 mL) three times. The EtOAc extract was dried to yield **7** (3.66 g, 102%): TLC $R_f = 0.41$, CH_2Cl_2/MeOH/CH_3COOH (5:1:0.001); ^{1}H NMR (500 MHz, CD_3OD) δ 3.77 (3H, s), 4.39 (2H, s), 6.71 (1H, d, $J = 15.5$ Hz), 6.88 (2H, d, $J = 8.6$ Hz), 6.98 (1H, d, $J = 15.5$ Hz), 7.22 (2H, d, $J = 8.6$ Hz); LC(ESI)MS $m/z = 236$ $[M + H]^+$.

(*S*)-*tert*-Butyl 2-((S)-3-(((9H-fluoren-9-yl)methoxy)carbonylamino)-2-(*tert*-butoxycarbonylamino)propanamido)-3-methylbutanoate (8). To couple DAP and L-valine, Boc-DAP(Fmoc)-OH (1 mmol, Bachem), Val-*t*Bu (1 mmol, Bachem), benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (1.1 mmol), and *N*,*N*-diisopropylethylamine (DIEA) (3 mmol) were dissolved in 10 mL of CH_2Cl_2 and stirred for 2 h. After the reaction had gone to completion (based on the disappearance of the DAP and L-valine starting materials in the LC-MS profile) CH_2Cl_2 was added to 100 mL and extracted with H_2O (100 mL), H_2O acidified with citric acid (100 mL), H_2O (100 mL), and then aqueous $NaHCO_3$ (100 mL). The CH_2Cl_2 was dried and then purified by flash chromatography on a silica column using EtOAc/hexanes (3:2) as an eluent to afford **8** (0.87 mmol, 87%): TLC $R_f = 0.75$, EtOAc/hexanes (3:2); ^{1}H NMR (500 MHz, CD_2Cl_2) δ 0.89 (3H, d, $J = 6.9$ Hz), 0.91 (3H, d, $J = 6.9$ Hz), 1.44 (9H, s), 1.46 (9H, s), 2.15 (1H, m), 3.54 (2H, t, $J = 5.3$ Hz), 4.22 (1H, t, $J = 6.8$ Hz), 4.33 (1H, dd, $J = 4.6$, 8.5 Hz), 4.39 (2H, t, 7.4 Hz), 5.60 (1H, br s), 5.68 (1H, br s), 6.89 (1H, br s), 7.32 (2H, t, $J = 7.4$ Hz), 7.40 (2H, t, $J = 7.4$ Hz), 7.61 (2H, d, $J = 7.5$ Hz), 7.78 (2H, d, $J = 7.5$ Hz); LC(ESI)MS $m/z = 582$ $[M + H]^+$.

(*S*)-*tert*-Butyl 2-((*S*)-3-amino-2-(*tert*-butoxycarbonylamino)propanamido)-3-methylbutanoate (9). The Fmoc protecting group of **8** was removed to enable coupling with **7** by dissolving **8** (0.87 mmol) in a solution of 10% piperidine in dimethylformamide (5 mL) and stirring at room temperature for 2 h. CH_2Cl_2 was added to the reaction mixture, and it was extracted with saturated aqueous $NaHCO_3$ (75 mL). The organic layer was dried and then purified by silica flash chromatography with CH_2Cl_2/MeOH (7:1) as an eluent. The fractions containing **9** were dried to yield an oil: TLC $R_f = 0.36$, CH_2Cl_2/MeOH (7:1); ^{1}H NMR (500 MHz, CD_2Cl_2) δ 0.89 (3H, d, $J = 6.9$ Hz), 0.92 (3H, d, $J = 6.9$ Hz), 1.44 (9H, s), 1.45 (9H, s), 2.16 (1H, m), 2.75 (1H, dd, $J = 7.8$, 12.1 Hz), 3.21 (1H, dd, $J = 2.8$, 12.2 Hz), 3.99 (1H, m), 4.33 (1H, dd, $J = 4.6$, 8.8 Hz), 5.65 (1H, br s), 7.69 (1H, br s); LC(ESI)MS $m/z = 360$ $[M + H]^+$.

(*S*)-*tert*-Butyl 2-((*S*)-2-(*tert*-butoxycarbonylamino)-3-((*E*)-4-(4-methoxybenzylamino)-4-oxobut-2-enamido)propanamido)-3-methylbutanoate (10). The dipeptide **9** was coupled with **7** using PyBop and DIEA. The product **9** from the previous reaction (approximately 0.86 mmol) was dissolved in CH_2Cl_2 (8 mL) along with **7** (0.86 mmol), PyBop (0.95 mmol), and DIEA (2.6 mmol). After stirring the reaction mixture for 2 h the CH_2Cl_2 was washed with H_2O acidified with 5% citric acid (pH 4), H_2O, and then saturated aqueous $NaHCO_3$. The CH_2Cl_2 was dried and purified by silica flash chromatography with CH_2Cl_2/MeOH (9:1) as an eluent to afford **10** (0.82 mmol, 95%): TLC $R_f = 0.31$, EtOAc; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (3H, d, $J = 6.5$ Hz), 0.88 (3H, d, $J = 5.7$ Hz), 1.41 (9H, s), 1.43 (9H, s), 2.13 (1H, m), 3.26 (1H, br s), 3.68 (2H, br s), 3.75 (3H, s), 4.32 (2H, dd, $J = 4.6$, 8.5 Hz), 4.38 (2H, brs), 5.88 (1H, br s), 6.54 (1H, br s), 6.82 (2H, d, $J = 8.5$ Hz), 6.95 (2H, br s), 7.16 (2H, d, $J = 8.3$ Hz); LC(ESI)MS $m/z = 577$ $[M + H]^+$.

(S)-*tert*-Butyl 2-((*S*)-3-((*E*)-4-amino-4-oxobut-2-enamido)-2-(*tert*-butoxycarbonylamino)propanamido)-3-methylbutanoate (11). The methoxybenzyl group was removed from **10** to form the amide by dissolving **10** (0.82 mmol) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)[22] (1.2 mmol) in CH_2Cl_2/H_2O (25:1), and the reaction was stirred overnight. CH_2Cl_2 (200 mL) was added to the reaction, washed twice with H_2O (200 mL), and then washed with saturated aqueous $NaHCO_3$ (200 mL). The CH_2Cl_2 was dried and the product was purified by silica flash chromatography using CH_2Cl_2/MeOH (5:1) as an eluent to yield **11** (0.37 mmol, 45%): TLC $R_f = 0.53$, CH_2Cl_2/MeOH (5:1); ^{1}H NMR (500 MHz, CD_3OD) δ 0.96 (6H, d, $J = 6.8$ Hz), 1.44 (9H, s), 1.48 (9H, s), 2.16 (1H, m), 3.49 (1H, dd, $J = 8.2$, 13.5 Hz), 3.64 (1H, dd, $J = 5.1$, 13.7 Hz), 4.22 (1H, d, $J = 5.4$ Hz), 4.34 (1H, dd, $J = 5.3$, 7.8 Hz), 6.88 (1H, d, $J = 15.2$ Hz), 6.93 (1H, d, $J = 15.3$ Hz); LC(ESI)MS $m/z = 457$ $[M + H]^+$.

Dapdiamide A (1). The final step in the synthesis of dapdiamide A is the removal of the Boc and *t*Bu protecting groups. **11** (0.37 mmol) was dissolved in CH_2Cl_2 (3 mL), TFA was added to 20%, and the reaction was stirred at room temperature for 24 h. After addition of H_2O (40 mL), the reaction was extracted twice with CH_2Cl_2 (40 mL). The aqueous layer was dried to yield dapdiamide A (0.34 mmol, 91%). ^{1}H and 2D NMR data of synthetic dapdiamide A were identical to dapdiamide A isolated from A10A. Dapdiamide A was tested against *E. amylovora*, and it was active in a similar concentration range as the isolated dapdiamide A.

Dapdiamide A (1): $[\alpha]^{23}_D$ +5.9 (isolated) and +9.5 (synthetic) (H_2O); ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; HRMS *m/z* 301.1499 $[M + H]^+$ (isolated) and 301.1504 $[M + H]^+$ (synthetic) (calcd for $C_{12}H_{21}N_4O_5$, 301.1512).

Dapdiamide B (2): ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; HRMS *m/z* 315.1656 $[M + H]^+$ (calcd for $C_{13}H_{23}N_4O_5$, 315.1668).

Dapdiamide C (3): ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; HRMS *m/z* 315.1666 $[M + H]^+$ (calcd for $C_{13}H_{23}N_4O_5$, 315.1668).

Dapdiamide D (4): ^{1}H and ^{13}C NMR spectroscopic data, see Table 2; HRMS *m/z* 301.1509 $[M + H]^+$ (calcd for $C_{12}H_{21}N_4O_5$, 301.1512).

Dapdiamide E (5): ^{1}H and ^{13}C NMR spectroscopic data, see Table 2; HRMS *m/z* 317.1461 $[M + H]^+$ (calcd for $C_{12}H_{21}N_4O_6$, 317.1461).

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Supporting Information Available: ^{1}H and 2D NMR spectra for **1**−**5**, map of transposon insertions, and table of sequence homologues. This information is available free of charge via the Internet at http://pubs.acs.org.

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